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(54) Title: MODIFICATION OF NITRIC OXIDE ACTIVITY TO TREAT FAS-INDUCED PATHOLOGIES

(57) Abstract

The present invention is directed to methods for treating pathological conditions associated with abnormalities in Fas-induced apoptosis by administering agents that either increase or decrease the levels of nitric oxide in cells.

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MODIFICATION OF NITRIC OXIDE ACTIVITY TO TREAT FAS-INDUCED PATHOLOGIES

Field of the Invention

The present invention is directed to methods that are useful in treating pathological conditions associated with abnormalities in Fas-induced apoptosis. The methods involve administering either nitric oxide donors or nitric oxide inhibitors to patients so as to modify Fas activity.

Background of the Invention

Apoptosis, also called "programed cell death," is a process by which excess, autoreactive, or damaged cells are removed from the body and tissues maintain homeostasis. However, dysregulation of apoptosis results in the abnormal accumulation or destruction of cells and is involved in the pathogenesis of a variety of disorders including autoimmune disease, cancer and acquired immunodeficiency syndrome (AIDS) (Thompson, *Science 267*:1456–1462 (1995)). One pathway controlling apoptosis is regulated by the cell surface receptor Fas, a member of the tumor necrosis receptor family. Fas induces apoptosis when bound by its natural ligand (expressed predominantly on activated T cells and natural killer (NK) cells) or by an agonist antibody (Itoh, *et al., J. Biol. Chem. 208*:10932–10937 (1993); Trauth, *et al., Science 245*:301–305 (1989)). Fas is expressed in the thymus, liver, heart, kidney, lymphoid and non-lymphoid malignancies and cell lines (Itoh, *et al., Cell 66*:233–243 (1991); Nagata, *Cell 88*:355–365 (1997)).

Fas has been found to be important to a number of physiological processes. Fas-induced apoptosis plays a critical role in modulating the immune response and humans deficient in Fas or the Fas ligand develop lymphoproliferative and autoimmune disorders (Drappa, *et al., Proc. Nat'l Acad. Sci. USA 90*:10340–10344 (1993); Fisher, *et al., Cell 81*:935–946 (1995); Rieux-Laucat, *et al., Science 268*:1347–1349 (1995)). This suggests a physiological role in the elimination of excess and/or autoreactive lymphocytes. In addition, activated or autoreactive T and B cells in the periphery are eliminated via Fas-induced apoptosis as are inflammatory cells entering immune privileged sites such as the eye and testis. Finally, cytotoxic T cells and

NK cells kill their targets via Fas-induced apoptosis (Arase, *et al.*, *J. Exp. Med. 181*:1235–1238 (1995); Ju, *et al.*, *P. Roc. Nat'l Acad. Sci. USA 91*:4185–4189 (1994)).

Many Fas-expressing cells are not sensitive to Fas-induced apoptosis, suggesting that cellular factors exist which inhibit signaling. Possible mediators of activity include elevated Bcl-2 or Bcl-xL expression (Rodriguez, *et al.*, *J. Exp. Med. 183*:1031–1036 (1996)); production of soluble Fas which blocks ligand binding (Cheng, *et al.*, *Science 263*:1759–1762 (1994)); or over-expression of FAP-1, a phosphatase which binds to Fas and inhibits apoptosis (Sato, *et al.*, *Science 268*:411–415 (1995)). Another potential inhibitor of Fas signaling is nitric oxide (NO), a small highly reactive biological messenger synthesized by the enzyme NO synthase (NOS) (Nathan, *et al.*, *Cell 78*:915–918 (1994)). A greater understanding of the importance of Fas in specific pathological states and of the way in which Fas activity can be regulated will help in determining when, and if, a patient may be effectively treated by modulating Fas activity.

Summary of the Invention

Although nitric oxide inhibitors and donors have been used in the treatment of inflammatory diseases and cancer, their mechanism of action has not been understood and clinical results have been highly variable. The present invention is based upon the discovery that these agents act by modulating Fas-induced apoptosis and that it is only the subset of patients whose maladies are associated with abnormalities in Fas activity that should be treated by their administration.

The invention is directed to a method of treating a patient for a pathological condition determined to be accompanied by an abnormality in Fas-induced apoptosis by administering an agent that alters the cellular activity of nitric oxide. Fas induced apoptosis is defined as being abnormal whenever it is greater or less than the activity seen in healthy individuals to a degree that is statistically significant ($p<0.05$). The best parameter for determining whether there is a Fas related abnormality is by staining and microscopically examining samples from a patient to see if the percentage of cells with apoptotic morphology is significantly different from the percentage in similar samples from normal individuals. In cases where a relationship has been established between a disease and abnormal Fas activity, merely assaying apoptotic activity to

determine if it is abnormally elevated or decreased is sufficient to decide whether a patient should be treated. Alternatively, a decision as to whether to treat a patient may be based upon assays for Fas expression or assays designed to determine Fas ligand concentration or activity.

In cases where a pathological condition is accompanied by abnormally elevated Fas-induced apoptosis, a patient is administered a nitric oxide donor compound at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with the pathological condition. A relationship has been established between abnormally elevated Fas activity and neoplastic diseases such as malignant melanoma. Other diseases that have been associated with abnormally elevated Fas-induced apoptotic activity include AIDS, diabetes mellitus, and Hashimoto's thyroiditis. Preferred nitric oxide donor compounds for treating such diseases are S-nitroso-N-acetylpenicillamine and sodium nitroprusside.

In cases where a pathological condition is accompanied by abnormally decreased Fas-induced apoptosis, a patient is administered a nitric oxide synthase inhibitor compound at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with the pathological condition. Abnormally decreased Fas activity has been associated with neoplastic disease. Specific diseases include colon cancer, renal cell carcinoma, hepatocellular carcinoma, breast cancer, non-Hodgkin's lymphoma, arthritis and nephritis. The preferred nitric oxide synthase inhibitor is N^G- monomethyl-L-arginine.

In a preferred method of treating a patient for a Fas-related pathological condition, one or more biological samples (*e.g.*, samples of tissue, blood, synovial fluid etc.) are obtained from an individual diagnosed as having a pathological condition that may be Fas-related and assayed for Fas expression and/or apoptotic activity. Fas expression can be determined from binding assays which utilize monoclonal antibodies to Fas or, alternatively, the percentage of Fas-positive cells in a sample can be quantitated using techniques such as flow cytometry (see Examples section). Apoptotic activity can be assessed by staining samples with acridine orange or some similar stain and then examining the sample microscopically to determine the percentage of cells with apoptotic morphology, i.e. nuclear and cytoplasmic condensation, nuclear fragmentation, membrane blebbing or apoptotic body formation. The results are compared with results obtained using samples from normal individuals in order to determine

whether the individual being tested has a condition that is "Fas-related." As used herein, a "Fas-related" pathological condition is one in which the patient has a level of Fas expression or apoptotic activity that differs from that of a normal individual to a statistically significant extent, $p<0.05$.

In its first aspect, the preferred method involves treating a patient for a pathological condition associated with excess Fas-induced apoptotic activity by administering a nitric oxide donor compound. Patients are selected for treatment either based on assays showing a level of Fas expression significantly greater than normal ($p<0.05$) or, preferably, based on assays showing significantly elevated apoptotic activity ($p<0.05$). The donor compound should be administered at a dosage and for a period of time sufficient to eliminate or substantially reduce one or more symptoms associated with the pathological condition. Conditions that can be treated by this method include Fas-related neoplastic disease (*e.g.*, malignant melanoma), AIDS, diabetes mellitus and Hashimoto's thyroiditis. In the case of neoplastic disease, the dosage of donor compound should, typically, be sufficient to retard abnormal cellular growth or reduce tumor mass. In the case of AIDS, donor compound should be administered at a dosage sufficient to improve the capacity of the patient for immune responsiveness. Donor compounds that have been found suitable for use in the method include S-nitroso-N-acetylpenicillamine and sodium nitroprusside. Alternative methods that produce the same overall result, *i.e.* an increase in cellular levels of NO, could also be employed. For example, gene transfer procedures which lead to an increase in cellular NOS could be used.

In a second aspect, the preferred method involves treating a patient for a pathological condition associated with abnormally low levels of Fas-induced apoptosis by administering a nitric oxide synthase inhibitor compound to the patient. Patients are selected for such treatment either based on assays showing a level of Fas expression significantly lower than normal ($p<0.05$) or, preferably, based on assays showing significantly reduced apoptotic activity ($p<0.05$). The NOS inhibitor should be administered at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with the pathological condition. Diseases that can be treated include Fas-related neoplastic disease, particularly lymphomas (*e.g.*, non-Hodgkin's lymphoma), colon cancer, renal cell carcinoma, hepatocellular carcinoma, breast cancer, and Fas-related autoimmune disease such as arthritis r nephritis. In

the case of arthritis and nephritis, NOS inhibitor should typically be given at a dosage sufficient to reduce pain, reduce cartilage damage, or improve kidney function. One compound that has been found to be effective in inhibiting Fas-induced activity is N^G-monomethyl-L-arginine. Other compounds can also be used, as can methods directed to inhibiting the expression of the NOS gene. For example, antisense oligonucleotides complimentary to a portion of the NOS gene may be introduced into cells to inhibit gene expression.

The invention is also directed to a method of regulating caspase activity in a patient, by administering an agent that alters the cellular activity of nitric oxide. The agent is administered at a dosage and for a duration sufficient to significantly alter caspase S-nitrosylation, *i.e.*, the percentage of nitrosylated caspase molecules is increased or decreased to a statistically significant extent. Examples of agents that can be used include nitric oxide donor compounds and nitric oxide synthase inhibitors.

Brief Description of the Figures

Figure 1: Expression of iNOS protein in human leukocytic cell lines: Whole cell lysates were made from 5 x 10^5 cells derived from a variety of human B cell, T cell or monocytic cell lines. A cell lysate made from the B958 marmoset B cell line was used as a control (B958). BJAB is an EBV-negative B lymphoma cell line; 10C9 and 2F7 are AIDS-related Burkitt's lymphoma B cell lines; U937 is a human promonocytic cell line; H9 is a human T lymphoma cell line; and Jurkat is a human T leukemia cell line. iNOS expression was detected using an affinity purified rabbit antiserum raised against the N-terminal 20 amino acids of the rat isoform, which recognizes both human and rat iNOS (Kobzik, *et al.*, *Am. J. Respir. Cell. Mol. Biol.* 9:371–377 (1993)).

Figure 2: NOS inhibits Fas-induced apoptosis in Burkitt's lymphoma cells: Panel A: BJAB cells were grown in the absence (control) or presence of the NOS inhibitor L-NMA (N); Fas-agonist antibody (F); Fas-agonist antibody together with L-NMA (FN); Fas-agonist antibody, L-NMA and L-arginine (FNL); or Fas-agonist antibody, L-NMA, and S-nitroso-penicillamine (FNS). After 24–48 hours, the percentage of cells with apoptotic morphology was determined by acridine orange staining. The results represent the mean +/− SEM of 6–24 separate experiments. * indicates p=0.001 versus F, † indicates p=0.03, Λ indicates p=0.004

versus FN. Panel B: BJAB cells were grown for 48 hours in the presence of media alone (control), Fas-agonist antibody (FAS), Fas-agonist antibody in combination with L-NMA (FAS + L-NMA), or L-NMA alone (L-NMA). The cells were then stained with propidium iodine and analyzed by flow cytometry. Ap indicates the apoptotic population. The results are representative of one of four separate experiments. Panel C: The same experiment was performed as in (A) using 10C9 cells. The results indicate the mean +/- SEM of 6–25 separate experiments. * indicates p=0.001 versus F, † indicates p=0.05, Λ indicates p=0.02 versus FN. The results were confirmed by DNA laddering analysis (data not shown).

Figure 3: NOS inhibits Fas-induced apoptosis in multiple cell lines of hematopoietic lineage: The T leukemia cell line Jurkat, the promonocytic cell line U937, the EBV transformed lymphoblastoid cell line IB4, the T lymphoma cell line H9 and the AIDS-related Burkitt's lymphoma cell line 2F7 were grown in the presence or absence of Fas-agonist antibody and the NOS inhibitor L-NMA as described in Figure 2. After 24–48 hours the percentage of apoptotic cells was determined by acridine orange staining. The data presents the mean +/- SEM of 3–6 separate experiments. * indicates p=0.05 (Jurkat), p=0.009 (U937), p=0.04 (IB4), p=0.007 (H9) and p=0.007 (2F7) versus F. Results were analyzed using a paired t test.

Figure 4: NOS inhibits Fas-induced apoptosis via a cGMP-independent mechanism: 10C9 cells were grown in the presence or absence of Fas-agonist antibody (10 ng/ml), L-NMA (5 mM), 8-bromo-cGMP (0.1-1.0 mM), Ly 83583 (10 μM) or ODQ (100 μM). After 24–48 hours the percentage of cells with apoptotic morphology was assessed by acridine orange staining. The results represent the mean +/- SEM of 2-11 separate experiments. 8-bromo-cGMP did not cause a statistically significant decrease in apoptosis induced by Ly 83583, ODQ or the combination of Fas and L-NMA. * indicates p=0.0001, Λ indicates p=0.0004 versus FN; † indicates p=0.009, # indicates p=0.03 versus control.

Figure 5: NOS does not alter Fas expression levels: H9 T cells were cultured in the presence (L-NMA) or absence (control) of the NOS inhibitor L-NMA (5 mM versus 0.5 mM L-arginine in the medium). After 48 hours, cells were stained with mouse anti-human Fas monoclonal antibody, followed by FITC-conjugated goat anti-mouse Ig; or with secondary

antibody alone; and analyzed by flow cytometry. Similar results were obtained using 10C9 B cells.

Figure 6: NOS inhibits Fas-induced PARP cleavage: Whole cell lysates were made from 5×10^5 10C9 cells grown for 2.5, 24 or 48 hours in the absence (control) or presence of Fas-agonist antibody (6 ng/ml) alone or in combination with L-NMA (5 mM versus 0.5 mM L-arginine in the medium). PARP and its 85 kD cleavage fragment were detected by Western blot analysis using a PARP-specific antiserum (Upstate Biotechnology). Similar results were seen using the H9 T cell line.

Figure 7: Intracelluar NO Production Inhibits Caspase-3-like Activity. 10C9 cells were left untreated (control) or were grown for 24 hours in the presence of the NOS inhibitor L-NMA to inhibit intracellular NO production. The cells were then cultured for 1.5 hours in the presence or absence of Fas agonist antibody (100 ng/ml) clone CH-11, Upstate Biotech). Caspase-3-like activity in cytosolic extracts prepared from these cells was measured with Ac-DEVD-pNA (200 uM) as described previously. Absorbance of released pNA was read at 405 nm at the indicated times and results are expressed as absorbance per mg of protein. Similar results were obtained with Jurkat cells.

Detailed Description of the Invention

Programed cell death, apoptosis, is a normal aspect of animal development and tissue homeostasis. The process serves to regulate cell number, facilitate morphogenesis, remove harmful or abnormal cells and eliminate cells that have already performed their function. Unfortunately, abnormal changes in the rate of cellular apoptosis sometimes occur and have been associated with a number of pathological conditions. The present invention is concerned with pathological conditions associated with abnormal levels of Fas-induced apoptosis and the treatment of such conditions by agents which serve to increase or decrease Fas-induced activity *in vivo*. More specifically, it has been found that nitric oxide is a natural inhibitor of Fas-induced activity and that by increasing or decreasing the levels of nitric oxide Fas-induced apoptosis can be regulated.

Diseases associated with excessive Fas-induced apoptosis, including malignant melanoma, AIDS, diabetes mellitus and Hashimoto's thyroiditis, may be treated by any method which produces an increase in cellular levels of nitric oxide *in vivo*. Preferably, this can be accomplished by administering a nitric oxide donor compound to the patient. Donor compounds that have been found suitable are S-nitroso-N-acetylpenicillamine and sodium nitroprusside. The total dosage of agent to be administered to a patient should be at least the amount required to reduce or eliminate symptoms associated with the disease being treated. For example, a patient being treated for Fas-related neoplastic disease should be given sufficient compound to retard or reverse abnormal cellular growth. Similarly, patients with Fas-related AIDS should be given sufficient compound to produce an increase in factors associated with the capacity of the patient for immunological responsiveness.

Physicians may begin by administering relatively small doses of agent and then adjust the dosage upward as it becomes clear that the patient can tolerate the treatment. For example, a physician may begin by administering 1 nmol/kg/day and increase the dosage to 1 μmol/kg/day, using parameters such as blood pressure as a guide. The final dosage may be provided in either a single or multiple regimen with the latter being generally preferred. These are simply guidelines since the actual dose must be carefully selected and titrated by the attending physician based upon clinical factors unique to each patient. The optimal daily dose will be determined by methods known in the art and will be influenced by factors such as the age of the patient, disease state, side effects associated with the particular agent being administered and other clinically relevant factors. In many cases, a patient will already be taking medications at the time treatment with the nitric oxide donor compound is initiated. These other medications may be continued during the time that the donor agent is administered to the patient but it is particularly advisable in such cases to begin with low doses to determine if adverse side effects are experienced.

Similar considerations apply to the treatment of Fas-related diseases that are characterized by abnormally low levels of Fas-induced apoptosis. Diseases such as Fas-related lymphoma and Fas-related autoimmune disease may be treated by any method compatible with *in vivo* administration resulting in decreased intracellular levels of nitric oxide. Typically, this will be accomplished through the administration of a nitric oxide synthase inhibitor such as N^G-

monomethyl-L-arginine. The dosage to be administered to the patient can be determined in essentially the same manner as discussed above for nitric oxide donor compounds.

The present invention is not limited to any particular dosage form or route of administration. Although oral administration will generally be most convenient, the invention is compatible with parenteral, transdermal, sublingual, buccal or implantable routes of administration as well. Agents may be given in a substantially purified form or, preferably, as part of a pharmaceutical composition containing one or more excipients or flavoring agents. Compositions may also include other active ingredients for the treatment of patients. The preparations may be solid or liquid and take any of the pharmaceutical forms presently used in medicine, *e.g.*, tablets, gel capsules, granules, suppositories, transdermal compositions or injectable preparations.

The active ingredient or ingredients may be incorporated into dosage forms in conjunction with the vehicles which are commonly employed in pharmaceutical preparations, e.g. talc, gum arabic, lactose, starch, magnesium stearate, cocoa butter, acquiesce or non-aqueous solvents, oils, paraffin derivatives, glycols, etc. Methods for preparing appropriate formulations are well known in the art (see e.g., Remington's Pharmaceutical Sciences, 16th ed., A. Oslo ed., Easton, PA (1980)).

Other methods for increasing or decreasing intracellular levels of nitric oxide may also be employed. For example, levels of nitric oxide may be increased in cells by increasing the expression of a nitric oxide synthase gene. Typically, this would involve constructing a vector containing the gene operably linked to a promoter active in mammalian cells and then transferring this DNA into cells using a vehicle suitable for *in vivo* delivery (*see e.g.*, Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989)). For example, the gene may be operably linked to the CMV early promoter and introduced into cells by means of a recombinant retrovirus. Standard techniques for the production of appropriate retroviral vectors have been described in the art (see e.g. U.S. No. 5,532,220). A promoter and coding sequence are said to be "operably linked" if induction of promotor function results in the transcription of the coding sequence and if the nature of the linkag between the two sequences does not result in the introduction of a frame-shift mutation

or interfere with the ability f regulatory sequences to direct the expression f the coding sequence. In addition to the use of promoters, a wide variety of transcriptional and translational regulatory sequences can be employed in vectors.

In cases where the Fas-related disease being treated is characterized by an abnormally low level of Fas-induced apoptosis, vectors expressing antisense oligonucleotides that inhibit the expression of NOS may be introduced into cells. The antisense sequence will be found on the opposite strand of DNA from the strand transcribing the nitric oxide synthase gene. The antisense sequences should generally be a minimum of 15 nucleotides in length and bind to endogenous transcripts in a manner which inhibits NOS expression in a highly specific manner. Use of antisense nucleic acid to block gene expression is discussed in Lichtenstein, C. *Nature 333*:801-302 (1988).

In order to determine the effect of treatment on disease, patients should be evaluated on a regular basis over an extended period of time. It may take several weeks for the full therapeutic effect of a treatment to become apparent. The effect of treatment on apoptotic activity can be determined on biological samples obtained from the patient by staining the samples and examining them microscopically to look for morphological characteristic of programmed cellular death (see Examples section). By comparing the results obtained to those obtained in samples from normal individuals, conclusions concerning the effectiveness of treatment can be made. In cases where samples are easily obtained, these results can be used as a means for complimenting other clinical indications of disease control or progression.

Examples

Example 1: Regulation of Fas-Induced Apoptosis

The present example provides evidence indicating that Fas-induced apoptosis can be controlled by regulating the levels of nitric oxide within cells. It was found that the NOS inhibitor N^G-monomethyl-L-arginine significantly increases Fas-induced apoptosis and that this effect can be reversed using the nitric oxide donors S-nitroso-N-acetylpenicillamine or sodium nitroprusside.

A. **Materials and Methods**

Induction of Apoptosis: 100 µl of BJAB or 10C9 cells which had been fed 1-3 days previously and were growing logarithmically (approximately 500,000 cells/ml) in RPMI 1640 containing 1 mM L-arginine and 10% fetal bovine serum (FBS), were plated in 96 well plates. 100 µl of modified RPMI 1640 containing 10% FBS (GIBCO SelectAmine) were added to each well to bring the final L-arginine concentration to 0.5 mM. The Fas-agonist monoclonal antibody clone CH/11, IgM (Upstate Biotechnology) was added to appropriate wells at 5-10 ng/ml for BJAB cultures and 5-20 ng/ml for 10C9 cultures. L-NMA (5 mM versus 0.5 mM L-arginine in the medium), L-arginine (2.5-5 mM), or S-nitrosopenicillamine (10-100 µM) were added to the indicated wells. After 24–48 hours, the percent of apoptotic cells was determined by staining the cells with acridine orange as described below.

Analysis of Apoptosis by Acridine Orange Staining: 30 µl of cells from each culture were pelletted, and 10 µl of cell slurry was mixed with 10 µl of acridine orange (5 µg/ml) diluted in phosphate buffered saline. The percentage of cells with apoptotic morphology (nuclear and cytoplasmic condensation, nuclear fragmentation, membrane blebbing, apoptotic body formation) was then analyzed on a wet mount slide using a Zeiss Axioskop equipped with epifluorescence.

Analysis of Apoptosis by Propidium Iodine Staining: Cells were stained with propidium iodine as described previously (Nicoletti, *et al.*, *J. Immunol. Methods 139*:271-279 (1991)). The percentage of apoptotic cells was quantitated using a Facscan Flow Cytometer (Becton Dickinson). Fluorescence data was collected using logarithmic amplification, and necrotic debris and cellular aggregates were eliminated from the data by forward and right angle light scatter gates. Apoptotic cells were distinguished from non-apoptotic intact cells by their decreased DNA content as determined by lower propidium iodine staining intensity.

Western Blot Analysis: Whole cell lysates were made by boiling cells in sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 20% glycerol, 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by SDS/polyacrylamide gel (7%) electrophoresis, and transferred to nitrocellulose. Rabbit anti-iNOS (used at a 1:500 dilution) or rabbit anti-PARP (Upstate Biotechnology, 1 µg/ml) were used as primary antibodies for Western blots and horseradish

peroxidase-labeled goat-anti-rabbit Ig was used as a secondary antibody (Amersham). The blots were developed using enhanced chemiluminescence (ECN) as per the manufacturer's instructions (Amersham).

Analysis of Fas Expression Levels: Cells were incubated in the presence or absence of 2 μg/ml of Fas monoclonal antibody (clone CH-11, IgM, Upstate Biotechnology) for 60 minutes at 4°C, followed by 3 washes in ice cold phosphate buffered saline (PBS) containing 0.1% fetal bovine serum and sodium azide. The cells were then incubated with fluorescein-conjugated goat-anti-mouse Ig (Jackson Labs) for 45 minutes at 4°C, washed 3 times and resuspended in 1% formalin. The percentage of Fas-positive cells was quantitated using a Facscan Flow Cytometer (Becton Dickinson).

B. Results

NOS Activity Inhibits Fas-Induced Apoptosis: To confirm that human leukocytes are capable of synthesizing NO (or related species) intracellularly, iNOS expression (the NOS isoform expressed in murine leukocytes) was analyzed by Western blot analysis in multiple human leukocytic cell lines. Basal expression of iNOS was detected in all human B cell, T cell and monocytic cell lines tested (Figure 1). The effects of NOS on Fas-induced apoptosis was examined in BJAB, the human B cell line which in previous studies was found to contain the highest levels of basal NOS activity (*see*, Mannick, *et al. Cell 79*:1137-1146 (1994)). Fas was ligated on the surface of BJAB cells with anti-Fas monoclonal antibody (5-10 ng/ml clone CH-11, IgM, Upstate Biotechnology) in the presence or absence of the competitive NOS inhibitor N^G and monomethyl-L-arginine (LNMA). NOS inhibition significantly increased Fas-induced apoptosis (Figures 2A and 2B), suggesting that constitutive NOS activity attenuates Fas-mediated apoptosis.

Confirming that the effects of L-NMA were due to a specific inhibition of NOS, the NOS substrate L-arginine, or the NO donors S-nitroso-N-acetylpenicillamine (Figure 2A) and sodium nitroprusside (data not shown) partially reversed the inhibition. These findings were reproduced in another Burkitt's lymphoma cell line, 10C9, which is derived from a patient infected with human immunodeficiency virus type 1 (HIV-1). Again, inhibition of NOS by L-NMA significantly increased Fas-induced apoptosis in 10C9 cells (Figure 2C) and the effects were

reversed by both L-arginine and the NO donors S-nitroso-N-acetylpenicillamine (Figure 2C) and sodium nitroprusside (data not shown).

Experiments were conducted to determine whether the findings in human B lymphoma cells lines were generalized to other human cell lines of hematopoietic lineage. L-NMA increased Fas-induced apoptosis in the T lymphoma cell line H9, the T leukemia cell line Jurkat, the promonocytic cell line U937, the EBV+ lymphoblastoid cell line IB4, and in the EBV+ AIDS-related Burkitt's lymphoma cell line 2F7 (Figure 3). Thus, intracellular NOS activity may be a general mechanism by which transformed cells of hematopoietic lineage inhibit Fas-induced apoptosis.

NOS Inhibits Fas-Induced Apoptosis Via A cGMP-Independent Mechanism: Significant inhibitory effects of nitric oxide (NO) on platelets and smooth muscle derive from increases in cGMP levels due to activation of soluble guanylyl cyclase (Moncada, *et al. Pharmacol. Rev. 43*:109-142 (1991); Ignarro, *Annu. Rev. Pharmacol. Toxicol. 30*:535-560 (1990)). However, the inhibitory effects of NO on Fas-induced apoptosis appear to be cGMP-independent because the permeable cGMP analog, 8-bromo-cGMP (0.1-1 mM) failed to reverse the inhibitory effects of L-NMA on Fas-induced apoptosis (Figure 4). Although the guanylyl cyclase inhibitors 1H-[1,2,3]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and LY 83583 (10-100 μM) increased spontaneous as well as Fas-induced apoptosis, the mechanism did not appear to be due to a specific inhibition of guanylyl cyclase, because the apoptosis was not reversed by 8-bromo-cGMP (0.1-1 mM) (Figure 4).

NOS Does Not Alter Fas Expression: One mechanism by which NOS could inhibit Fas-induced apoptosis is by decreasing the expression of Fas levels in cells. To test this hypothesis, 10C9 B cells or H9 T cells were grown in the presence or absence of the NOS inhibitor L-NMA and Fas expression was analyzed by immunofluorescent staining and flow cytometry. L-NMA had no effect on Fas expression at concentrations which increased Fas-induced apoptosis (Figure 5), indicating that other mechanisms are involved in the inhibitory effects of NOS.

NOS Inhibits Fas-Induced PARP Cleavage: Since NOS did not affect Fas expression levels, the steps in the Fas signaling path way which might be inhibited by NOS were mapped.

Members f the caspase family of cysteine proteases are activated by Fas ligation and cleave poly(ADP)ribose polymerase (PARP) to its signature 85 kD fragment (Nagata, *Cell 88*:355-365 (1997); Schlegel, *et al., J. Biol. Chem. 271*:1841-1844 (1996)). Caspase activation can be monitored by analyzing the extent of PARP cleavage in cells. To determine whether caspase activation is inhibited by NOS, 10C9 or H9 cells were grown in the presence or absence of Fas-agonist antibody and L-NMA. At various time intervals, the extent of PARP cleavage was assessed by Western blot analysis using anti-PARP polyclonal antibody. NOS inhibition resulted in increased levels of Fas-induced PARP cleavage (Figure 6), suggesting that NOS inhibits caspase activation.

C. Discussion

The results presented herein indicate that NOS inhibits Fas-induced apoptosis in human B cell, T cell and monocytic cell lines via a cGMP-independent mechanism. NOS appears to inhibit Fas-induced caspase activation and PARP cleavage without altering levels of Fas expression. Previous studies have shown that NO can exert either pro- or anti-apoptotic effects which appear to be cell and stimulus specific (Albina, *et al., J. Immunol. 157*:5080-5085 (1993); Beauvais, *et al., FEBS Lett. 361*:229-232 (1995); Blanco, *et al., Am. J. Pathol. 146*:75-85 (1995); Chun, *et al., Endocrinol. 136*:3120-3127 (1995); Genaro, *et al., J. Clin. Invest. 95*:1884-1890 (1995); and Mannick, *et al., Cell 79*:1137-1146 (1994)). The cellular factors and target proteins which are responsible for the specificity of NO-related effects on apoptosis remain to be determined.

The cGMP-independent actions of NO (*e.g.*, inhibition of Fas-induced apoptosis in human leukocytes) appear to occur through S-nitrosylation of proteins, or modulation of redox state. Both mechanisms may operate to inhibit Fas signaling. In addition, NO inhibits many enzymes, including cysteine proteases, by S-nitrosylation of active site thiols (Stamler, *et al., Proc. Nat'l Acad. Sci. USA 89*:444-448 (1992)). The observation that NOS inhibits Fas-induced apoptosis may explain a previously described association between NO and Fas in studies of the MRL lpr/lpr mouse. The MRL lpr/lpr mouse carries a spontaneous Fas mutation which impairs Fas transcription (Adachi, *et al., Proc. Nat'l Acad. Sci. USA 90*:1756-1760 (1993)). MRL lpr/lpr mice develop arthritis and nephritis, presumably as a result of impaired Fas-mediated apoptosis of autoreactive lymphocytes. However, other strains of mice carrying the same Fas

mutation do not develop autoimmune disease, suggesting that additional factors are involved in the phenotype (Izui, *et al.*, *J. Immunol. 133*:227-233 (1984)). Interestingly, NOS expression and NO production are increased in MRL lpr/lpr mice but not in B6 lpr/lpr mice (who do not develop arthritis or glomerulonephritis); and the glomerulonephritis and arthritis can be reduced in MRL lpr/lpr mice using NOS inhibitors (Weinberg, *et al.*, *J. Exp. Med. 179*:651-660 (1994)). The mechanism by which NO induced glomerulonephritis and arthritis in these mice has not been elucidated. However, the data presented herein raise the possibility that NO production may unmask the lpr phenotype by inhibiting already low levels of Fas-mediated apoptosis of autoreactive lymphocytes.

The failure of cells to undergo apoptosis is a factor contributing to the development of some forms of cancer (Thompson, *Science 267*:1456-1462) (1995)). Resistance to Fas-mediated apoptosis may contribute to the pathogenesis of lymphomas because many primary lymphoid neoplasms express Fas without undergoing apoptosis (Kondo, *et al.*, *Am. J. Pathol. 145*:330-337 (1994)). Elevated levels of NO production may contribute to Fas-resistance in some of these tumors. In support of this hypothesis, HIV-1 infected patients have a markedly increased incidence of non-Hodgkin's lymphoma (Neri, *et al.*, *Blood 77*:1092-1095) (1991)); and elevated levels of nitrates and nitrites (the stable end products of NO synthesis) in their blood (Bukrinsky, *et al.*, *J. Exp. Med. 181*:735-745 (1995); Evans, *et al.*, *Clin. Exp. Immunol. 97*:83-86 (1994); Torre, *et al.*, *AIDS 9*:979-980 (1995)). Thus, elevated NO production may inhibit apoptotic death of lymphoma cells in AIDS patients, and thereby contribute to lymphomagenesis. NO-based therapies should be useful in the treatment of these malignancies and other Fas-associated diseases.

Example 2: Fas-Induced Caspase Denitrosylation

Nitric oxide and related molecules may inhibit apoptosis through the S-nitrosylation of the catalytic site cysteine of caspase proteins, a family of cysteine proteases which execute the cell death program. The present example examines whether caspases are actually S-nitrosylated intracellularly and whether caspase S-nitrosylation/denitrosylation is an integral component of the apoptotic pathway.

Caspase-3 and caspase-8 were immunoprecipitated from four different human B and T cell lines. Caspase-3 and caspase-8 were efficiently immunoprecipitated with their respective antibodies but not with control antibody. Nitrosylation of the immunoprecipitated proteins was measured by photolysis/chemiluminescence assays. Approximately 30-120 nM of NO was detected and caspase-3 immunoprecipitates and 10-20 nM NO was detected in the caspase-8 immunoprecipitates. NO was not detected in control immunoprecipitates or in caspase-3 immunoprecipitates which had been pretreated with $HgCl_2$, a compound that selectively removes NO groups from S-nitrosothiol bonds. Therefore, all of the detectable NO in caspase-3 immunoprecipitates appears to be derived from S-NO bonds. By silver staining, the approximate concentration of caspase-3 and caspase-8 in the immunoprecipitates was equimolar with the concentration of NO released. Since data in cell free systems indicates that caspases are S-nitrosylated at a single cysteine, the results suggest that the majority of caspase-3 and caspase-8 is S-nitrosylated intracellularly.

In order to determine if the Fas signaling pathway altered the level of S-nitrosylation, caspase-3 was immunoprecipitated from cells which had been stimulated with Fas agonist antibody. 45 minutes (Jurkat) to 2 hours (10 C9) after Fas crosslinking, nitrosylated caspase was decreased by approximately 66%. However, only a minority of caspase-3 zymogen was cleaved to its active subunits at this time point, suggesting that Fas activation decreased the S-nitrosylation of caspase zymogen before it was cleaved to its active subunits.

The decline in S-nitrosylated caspase-3 could result from either a decrease in the rate of S-nitrosylation where a stimulation of denitrosylation activity. To distinguish between these possibilities, the extent of caspase-3 S-nitrosylation was analyzed in cells growth in the presence of the NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMA). Inhibition of intracellular NO production by L-NMA for 2 hours decreased caspase-3 S-nitrosylation by only 25%, although an approximate 50% decrease was noted at 18 hours. Thus, the rapid decline in caspase-3 S-nitrosylation 2 hours after Fas activation is likely the result of an increase in denitrosylation activity.

In order to determine if caspase S-nitrosylation is functionally coupled to intracellular caspase activity, caspase-3-like activity was measured in lysates of cells grown in the presence

or absence of L-NMA for 18 hours. Although N-NMA inhibited caspase S-nitrosylation by approximately 50 percent, this was not associated with an increase in caspase-3-like activity, suggesting that decreased S-nitrosylation alone is not sufficient to activate caspases. However, L-NMA increased Fas-induced caspase activation early after Fas crosslinking (Figure 7). Thus, caspase activation likely requires both the cleavage of the zymogen and the denitrosylation of the active site cysteine. After long periods of Fas activation (greater than or equal to 2 hours), L-NMA no longer increased Fas-induced caspase activity, probably because Fas alone had induced the denitrosylation of caspases at these time points.

The results suggest that intracellular NO production maintains caspase-3 and caspase-8 zymogens in an inactive, S-nitrosylated form in resting human cell lines. In addition, the results suggest that, upon activation of the Fas, apoptotic pathway, caspase zymogens are not only cleaved to their active subunits, but are also denitrosylated thereby freeing the catalytic site cysteine. Thus, it appears that protein S-nitrosylation/denitrosylation is a regulatory event in the Fas apoptotic pathway. The function of ion channels, G proteins, transcription factors and multiple enzymes can be altered by S-nitrosylation. The concept of S-nitrosylation being dynamically regulated and coupled to cell surface signals has implications for other signal transduction cascades and cellular control mechanisms.

All references cited are fully incorporated by reference. Having now fully described the invention, it will be understood by those of skill in the art that the invention may be performed within a wide and equivalent range of conditions, parameters, and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

What is Claimed is:

1. A method of treating a patient for a pathological condition determined to be accompanied by an abnormality in Fas-induced apoptosis, said method comprising administering to said patient an agent that alters the cellular activity of nitric oxide at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with said pathological condition.

2. The method of claim 1, wherein said pathological condition is accompanied by abnormally elevated Fas-induced apoptosis and said patient is administered a nitric oxide donor compound at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with said pathological condition.

3. The method of claim 2, wherein said pathological condition is a Fas-related neoplastic disease.

4. The method of claim 2, wherein said pathological condition is selected from the group consisting of: malignant melanoma, AIDS, diabetes mellitus, Hashimoto's thyroiditis, colon cancer, renal cell carcinoma, hepatocellular carcinoma, large granular lymphocytic leukemia and NK lymphoma.

5. The method of claim 2, wherein said nitric oxide donor compound is selected from the group consisting of: S-nitroso-N-acetylpenicillamine and sodium nitroprusside.

6. The method of claim 1, wherein said pathological condition is accompanied by abnormally decreased Fas-induced apoptosis and said patient is administered a nitric oxide synthase inhibitor compound at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with said pathological condition.

7. The method of claim 6, wherein said pathological condition is a Fas-related neoplastic disease.

8. The method of claim 6, wherein said neoplastic disease is a Fas-related lymphoma.

9. The method of claim 6, wherein said pathological condition is a Fas-related autoimmune disease.

10. The method of claim 6, wherein said pathological condition is selected from the group consisting of: colon cancer, renal cell carcinoma, hepatocellular carcinoma, breast cancer, non-Hodgkin's lymphoma, arthritis and nephritis.

11. The method of claim 6, wherein said nitric oxide synthase inhibitor is N^G- monomethyl-L-arginine.

12. A method of treating a patient for a Fas-related pathological condition, comprising:

a) obtaining a biological sample from an individual diagnosed as having a pathological condition that may be Fas-related;

b) assaying said biological sample for Fas expression and/or apoptotic activity;

c) comparing the results obtained in step b) with results obtained using samples from normal individuals;

d) identifying the individual of step a) as a patient with a Fas-related pathological condition if the comparison of step b) indicates a significantly greater than normal level of Fas expression or a significantly greater than normal level of apoptotic activity;

e) administering a nitric oxide donor compound to said patient at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with said pathological condition.

13. The method of claim 12, wherein said pathological condition is a Fas-related neoplastic disease.

14. The method of claim 12, wherein said Fas-related pathological condition is selected from the group consisting of: malignant melanoma, AIDS, diabetes mellitus, Hashimoto's thyroiditis,

col n cancer, renal cell carcinoma, hepatocellular carcinoma, large granular lymphocytic leukemia and NK lymphoma.

15. The method of claim 12, wherein said nitric oxide donor compound is selected from the group consisting of: S-nitroso-N-acetylpenicillamine and sodium nitroprusside.

16. A method of treating a patient for a Fas-related pathological condition, comprising:

a) obtaining a biological sample from an individual diagnosed as having a pathological condition that may be Fas-related;

b) assaying said biological sample for Fas expression and/or apoptotic activity;

c) comparing the results obtained in step b) with results obtained using samples from normal individuals;

d) identifying the individual of step a) as a patient with a Fas-related pathological condition if the comparison of step b) indicates a significantly lower than normal level of Fas expression or a significantly lower than normal level of apoptotic activity;

e) administering a nitric oxide synthase inhibitor compound to said patient at a dosage and for a duration sufficient to eliminate or substantially reduce one or more symptoms associated with said pathological condition.

17. The method of claim 16, wherein said pathological condition is a Fas-related neoplastic disease.

18. The method of claim 17, wherein said neoplastic disease is a Fas-related lymphoma.

19. The method of claim 16, wherein said pathological condition is a Fas-related autoimmune disease.

20. The method of claim 16, wherein said Fas-related pathological condition is selected from the group consisting of: colon cancer, renal cell carcinoma, hepatocellular carcinoma, breast cancer, non-Hodgkin's lymphoma, arthritis and nephritis.

21. The method of claim 16, wherein said nitric oxide synthase inhibitor is N^G- monomethyl-L-arginine.

22. A method of regulating caspase-induced apoptotic activity in a patient, comprising administering to said patient an agent that alters the cellular activity of nitric oxide, wherein said agent is administered at a dosage and for a duration sufficient to significantly alter caspase S-nitrosylation.

23. The method of claim 22, wherein said agent is a nitric oxide donor compound.

24. The method of claim 22, wherein said agent is a nitric oxide synthase inhibitor compound.

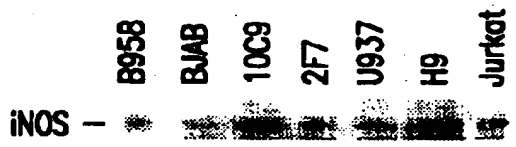

FIG.1

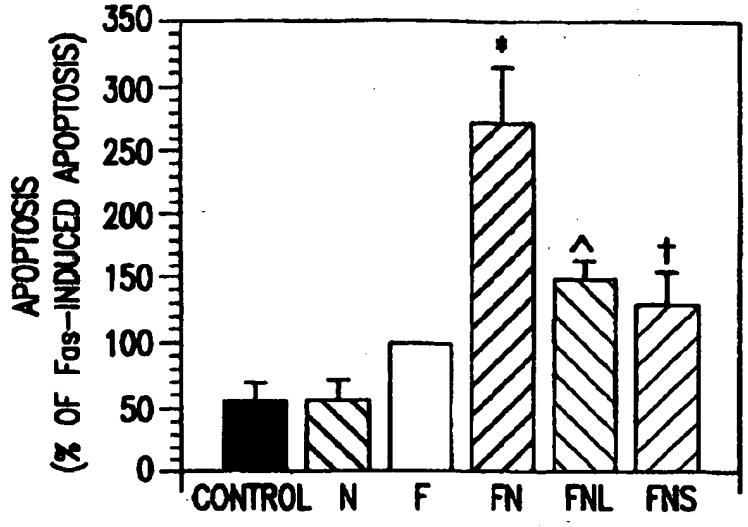
APOPTOSIS
(% OF Fas-INDUCED APOPTOSIS)
350
300
250
200
150
100
50
0
*
^
†
CONTROL
N
F
FN
FNL
FNS

FIG.2A

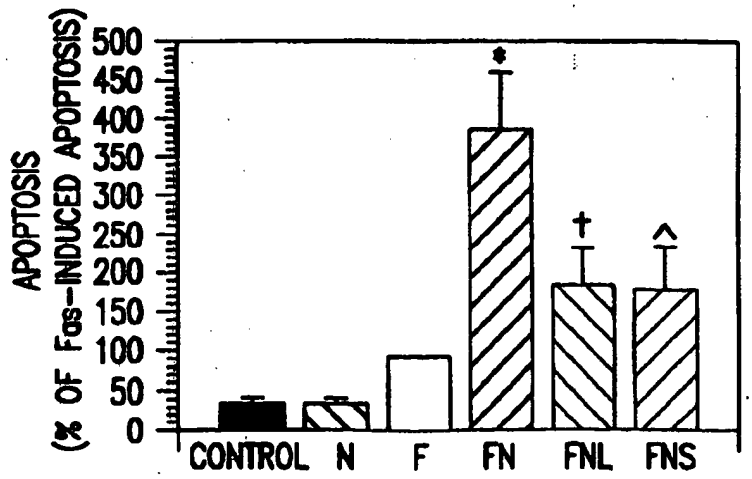
APOPTOSIS
(% OF Fas-INDUCED APOPTOSIS)
500
450
400
350
300
250
200
150
100
50
0
*
†
^
CONTROL
N
F
FN
FNL
FNS

FIG.2C

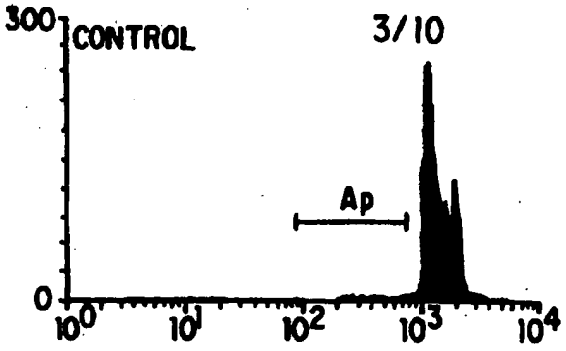

FIG. 2B-1

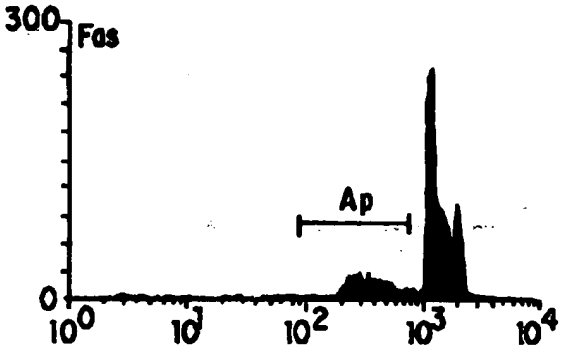

FIG. 2B-2

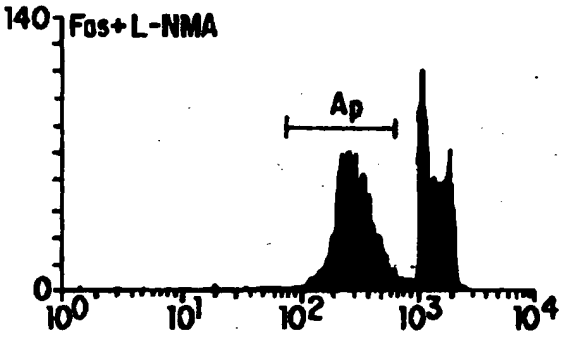

FIG. 2B-3

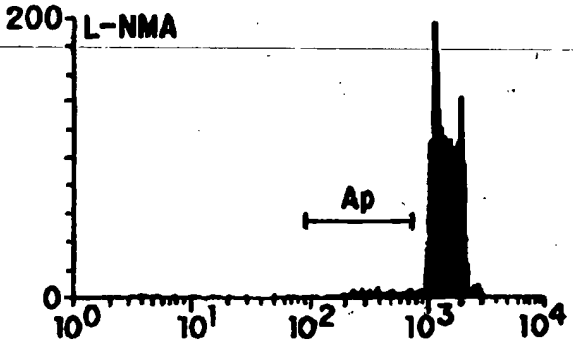

FIG. 2B-4

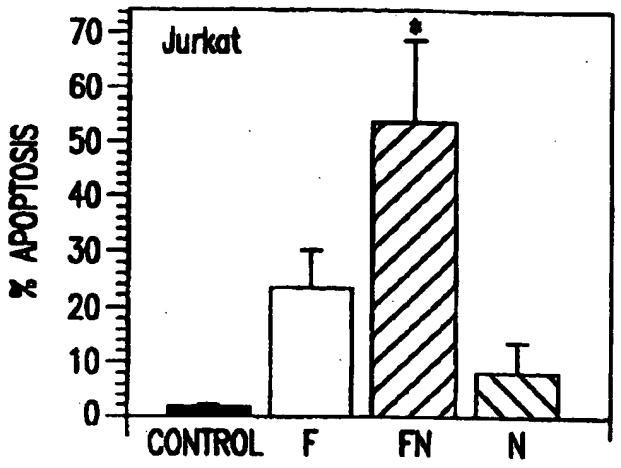
Jurkat
% APOPTOSIS
70
60
50
40
30
20
10
0
CONTROL
F
FN
N
*

FIG.3A

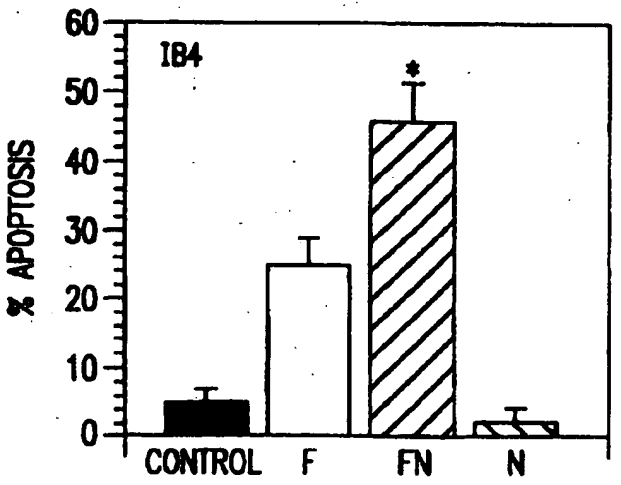
IB4
% APOPTOSIS
60
50
40
30
20
10
0
CONTROL
F
FN
N
*

FIG.3B

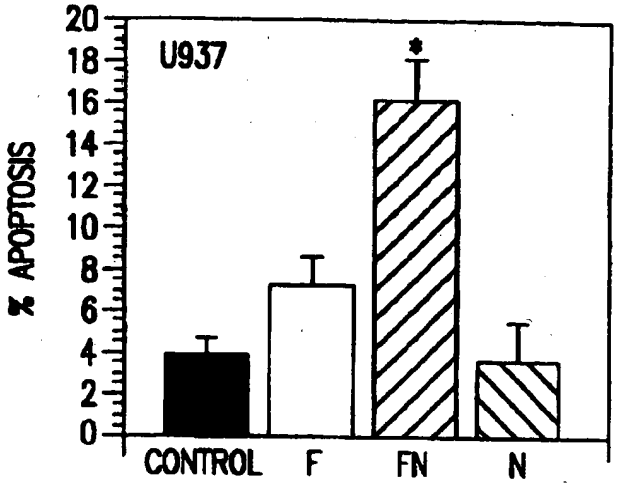
U937
% APOPTOSIS
20
18
16
14
12
10
8
6
4
2
0
*
CONTROL
F
FN
N

FIG.3C

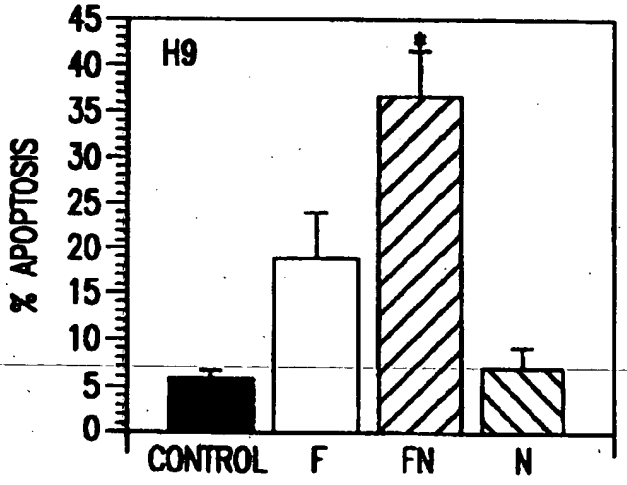
H9
% APOPTOSIS
45
40
35
30
25
20
15
10
5
0
*
CONTROL
F
FN
N

FIG.3D

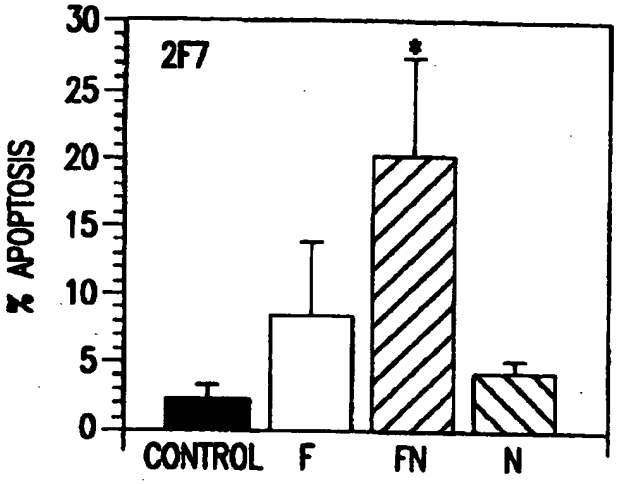

FIG.3E

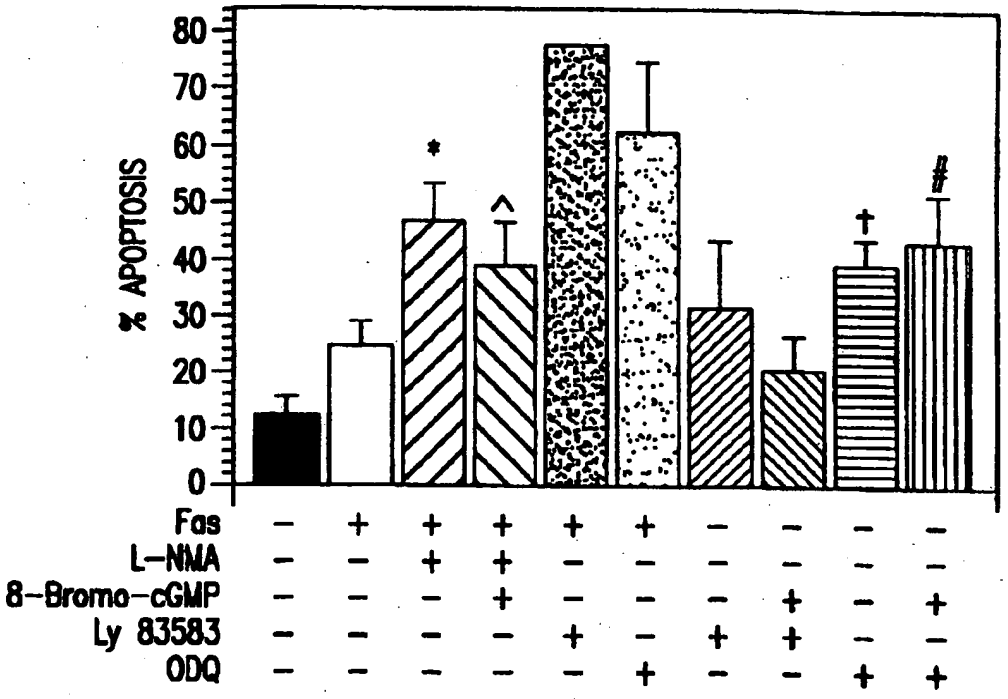
% APOPTOSIS
80
70
60
50
40
30
20
10
0
*
^
†
#
Fas − + + + + + − − − −
L-NMA − − + + − − − − − −
8-Bromo-cGMP − − − + − − − + − +
Ly 83583 − − − − + − + + − −
ODQ − − − − − + − − + +

FIG.4

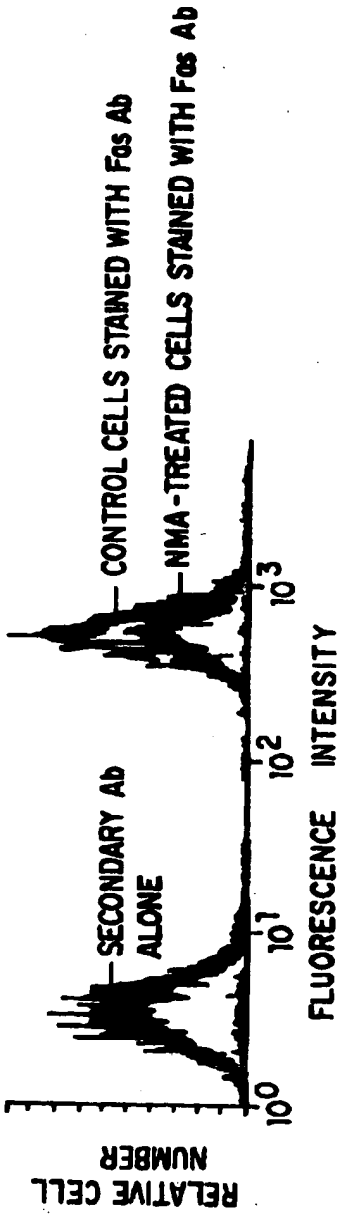
CONTROL CELLS STAINED WITH Fas Ab
NMA-TREATED CELLS STAINED WITH Fas Ab
SECONDARY Ab ALONE
10^0
10^1
10^2
10^3
FLUORESCENCE INTENSITY
RELATIVE CELL NUMBER

FIG. 5


2.5 HOURS
24 HOURS
48 HOURS
CONTROL
Fas
Fas + NMA
NMA
CONTROL
Fas
Fas + NMA
NMA
CONTROL
Fas
Fas + NMA
NMA
HeLa
PARP
85kD FRAGMENT

FIG.6

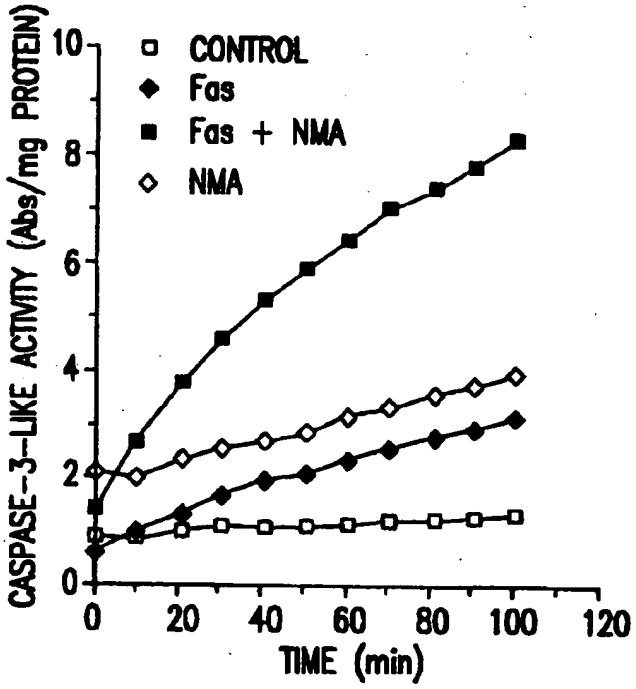
CASPASE-3-LIKE ACTIVITY (Abs/mg PROTEIN)
TIME (min)
CONTROL
Fas
Fas + NMA
NMA
0
2
4
6
8
10
0
20
40
60
80
100
120

FIG.7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/14457

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/295

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 419 901 A (GRIFFITH OWEN W) 30 May 1995 see column 3, line 39 - line 44 see column 3, line 57 - line 59 see column 4, line 2	1,6,9,11
X	US 5 554 638 A (DEWHIRST MARK W ET AL) 10 September 1996 see column 4, line 63 - line 65 see column 9, line 35	1,6,7,11
X	GARBAN ET AL: "nitric oxide up-regulates Fas receptor expression and sensitizes the AD10 ovarian carcinoma cell line to Fas-mediated apoptosis" PROC ANN. MEET AM ASS. CANCER RES., vol. 38, 1997, page 626 XP002080565 see abstract	1-3,5

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1998	27/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Trifilieff-Riolo, S

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/14457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ORUCEVIC ET AL: "effects of nma on IL-2 induced antitumor killer cell generation in vivo and in vitro" PROC ANN. MEET AM ASS. CANCER RES., vol. 35, 1994, page 521 XP002080566 see abstract	1,6,7,11
X	STEFANOVIC-RACIC ET AL: "comparison of the nitric oxide synthase inhibitors methylarginine and aminoguanidine as prophylactic and therapeutic agents in rat adjuvant arthritis" J. OF RHEUMATOLOGY, vol. 22, no. 10, 1995, pages 1922-1928, XP002080567 see abstract	1,6,9-11
X	WEINBERG ET AL: "the role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease" J EXP MED, vol. 179, no. 2, 1994, pages 651-660, XP002080568 cited in the application see abstract see page 658, right-hand column, line 10 - line 16	1,6,9-11
X	COCHRAN: "inhibition of nitric oxide synthesis as a therapeutic target for arthritis " EXPERT OPINION INVEST. DRUGS, vol. 3, no. 5, 1994, pages 529-531, XP002080569 see page 529, right-hand column, paragraph 2 see page 530, left-hand column, paragraph 1	1,6,9-11
X	JUN CHANG-DUK ET AL: "high-dose nitric oxide induces apoptosis in HL-60 human myeloid leukemia cells" EXP AND MOL MED, vol. 23, no. 2, 1996, pages 101-108, XP002080570 see abstract	1-5

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/14457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US 93340654, 1993 MCCARTNEY-FRANCIS: "suppression of arthritis by and inhibitor of nitric oxide synthase" XP002080571 see abstract	1,6,10, 11
X	& MCCARTNEY-FRANCIS ET AL: J EXP MED, vol. 178, no. 2, 1993, pages 749-754, see abstract	1,6,10, 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 14457

Box I Observations where certain claim were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-24 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No requires additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

National Application No
PCT/US 98/14457

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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